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(54) Proteases capable of shedding the soluble TNF-receptor and TNF-R derived peptides and antibodies against the proteases inhibiting the shedding.

(57) Molecules which influence the shedding of the cell-bound p55 Tumor Necrosis Factor receptor (p55-TNF-R), are provided, together with methods of producing them.
More particularly, the invention relates to proteases which cleave the cell-bound p55 TNF-R thus creating the soluble receptor and to inhibitors to these proteases comprising sequence asn-172 to thr-182 of p55-TNF-R or muteins thereof.

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The present invention relates to molecules which influence shedding of the cell-bound p55 Tumor Necrosis Factor receptor (p55 TNF-R), and to their preparation. More particularly, the invention relates to proteases which cleave the cell-bound p55 TNF-R thus creating the soluble receptor, to methods of preparing them, and to their use.

It is known that many cell-surface proteins occur also in soluble forms. Some of these soluble molecules are created as such **in vivo** from distinct mRNAs, transcribed by alternative splicing mechanisms from the same genes which encode the cell-surface proteins. Others are derived from the cell-surface proteins presumably by proteolytic cleavage or, in the case of lipid anchored proteins, by the cleavage of their lipid anchor. Shedding of cell-surface proteins may occur spontaneously and, for example cancer cells seem to have a propensity for doing so. Shedding may also be induced by various stimulating agents.

Knowledge of the mechanisms involved in the shedding of cell-surface proteins and in its regulation is quite limited. None of the proteases or lipases taking part in it have so far been identified. There is also no clear indication of the subcellular site at which it occurs - on the cell surface or within some other intracellular compartment such as the lisozomes to which the cell-surface proteins are transported.

We have now been able to shed light on the mechanisms by which a cell surface protein which serves as a receptor for a cytokine, the p55 TNF receptor, is shed by cells. There are two distinct receptors, the p55 and p75 receptors, by which TNF, a cytokine produced primarily by mononuclear phagocytes, initiates its multiple effects on cell function. Both receptors are expressed in many cell types yet in differing amounts and proportions. The variation in their amounts seems to affect significantly the nature and intensity of the cellular response to TNF. One of the ways by which their expression is regulated is through induced shedding of the receptors. They can be shed in response to different kinds of inducing agents, depending on the type of cells. Granulocytes, for example, shed both receptors in response to the chemotactic peptide - fMLP (formylmethionylleucylphenylalanine) and shed specifically their p75 receptor when treated by TNF, while in T lymphocytes shedding of the p75 receptor, which is the predominant TNF receptor species in these cells, occurs upon antigen stimulation.

Shedding of both receptors may also effectively be induced by PMA (phorbol myristate acetate), by the serine phosphate inhibitor okadeic acid and by the calcium ionophore-A23187. The effect of PMA could be shown to reflect activation of protein kinase C, while the effect of okadeic acid seemed to involve the function of some other serine kinase. The amino acid sequences of the soluble forms of the two receptors which had been isolated from urine, correspond to sequences of a cysteine-rich module which extends along a major part of the extracellular domain of the two cell surface receptors. The C terminus of the urine-derived soluble form (Nophar Y., et al., EMBO J., **Vol. 9**, No.10, pp. 3269-3278 (1990)) of the p55 receptors was initially defined as Asn 172 which is located 11 residues upstream to the transmembranal domain of this receptor, while the C terminus of the soluble form of the p75 receptor corresponds to the residue located 44 amino acid upstream to the transmembranal domain of this receptor. However it was later revealed, that in urine also a somewhat longer soluble form of the p55 receptor, extending two further amino acids downstream towards the intracellular domain exists (Wallach D., et al., Tumor Necrosis Factor III, (Eds. T. Osawa and B. Bonavida) S. Karger Verlag (Basel) pp47-57 (1991). Whether these C termini correspond to the sites at which the receptor had initially been shed upon its release from the cell surface, or reflects also some further cleavage of the soluble form, occurring in the serum or the urine, is yet unknown.

Besides the impact of the shedding of the TNF receptors on the amounts of the cell-surface expressed receptors, this process also seems to contribute to the control of TNF function through effects of the soluble forms of the receptors, which maintain the ability to bind TNF and in doing so can affect its function in two, practically opposing, manners. On the one hand they inhibit the function of TNF by competing for it with the cell-surface receptors but, on the other hand, have also a stabilizing effect on TNF and can thus prolong its effects. The soluble forms of both species of the TNF receptor occur in human serum at concentrations which are normally very low, yet increase dramatically in various disease states, apparently due to enhanced receptor shedding, reaching levels at which they can effectively modulate TNF function.

To gain knowledge of the mechanisms of shedding of the TNF receptors we are attempting to identify the structural elements within the receptors which are involved in their cleavage. Previously we examined the effect of cytoplasmic deletions on the function and shedding of the p55-TNF-R. We found that the signaling activity of the receptor depends on some function(s) of the C terminal part of the intracellular domain. However its shedding and the enhancement of the shedding by PMA occurs even in the complete absence of this domain (Brakebusch C., et al., EMBO J., **Vol 11**, pp. 943-950 (1992)).

The present invention provides a protease which is capable of cleaving the soluble TNF-R from the cell-bound TNF-R.

Preferably, the TNF-R is p55 TNF-R.

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The invention also provides a method for preparing a protease capable of cleaving the soluble TNF-R from the cell-bound TNF-R, comprising:

a) preparing a construct comprising an amino acid sequence inhibiting the protease,
b) affixing said construct to an affinity chromatography column,
c) passing a biological sample containing the protease through the column, and
d) recovering the protease from the column.

After isolation the protease is purified by conventional methods.

In one embodiment the above construct is prepared by known recombinant methods.

In another embodiment the construct comprises a synthetic peptide.

The invention also provides an antibody to the protease according to the invention which is capable of binding to the protease and either neutralizes the enzymatic activity of the protease or prevents the protease from binding to the receptor.

Such an antibody may either be polycolonal or monoclonal, and may be either murine or human, and may be prepared in a conventional manner.

The invention also provides a method for enhancing soluble TNF-R function, comprising administering an effective amount of a protease according to the invention to a patient.

In another aspect the invention provides a method for enhancing TNF function comprising administering an effective amount of an antibody according to the invention to a patient.

The invention also provides inhibitors of proteases comprising any of the following constructs depicted in Figure 5:

a) Δ 172-173
b) Δ 173-174
c) Δ 174-175
d) Δ 173
e) V 173 P
f) K 174 P
g) G 175 P
h) V 173 D
i) V 173 G

Such inhibitors may also be muteins of the above constructs.

The invention also provides a DNA molecule comprising a nucleotide sequence encoding the protease capable of cleaving the soluble TNF-R from the cell-bound TNF-R.

Furthermore, the invention provides DNA molecules hybridizing to said DNA molecule, preferably under stringent conditions, and encoding a protease with said biological activity.

A transformant host cell transformed with the replicable expression vehicle encoding the protease of the invention, which expression vehicle may be either prokaryotic or eukaryotic, also forms part of the present invention.

The protease in accordance with the invention is produced recombinantly by culturing a transformant host cell of the invention in a suitable culture medium and isolating the protease.

Pharmaceutical compositions comprising the protease of the invention as active ingredient together with a pharmaceutically acceptable carrier form yet another aspect of the invention.

Figure 1 shows the nucleotide and amino acid sequence of the p55 TNF-R, the transmembranal region being encircled, the cysteines being boxed and the putative glycosylation sites being overlined. The dashed overline indicates the N terminus of the soluble p55 TNF-R and the arrows indicate the major and minor C termini of the soluble p55 TNF-R.

Figure 2 is a diagrammatic presentation of human p55 TNF-R murine EGF receptor chimeric molecules used for studying the involvement of the transmembranal and intracellular domains of the p55 TNF-R in its shedding.

Figure 3 shows the results of a test of the ability of PMA to induce shedding of the chimeric molecules of Figure 2, in terms of the ability of the cells expressing them to bind radiolabeled TNF after PMA treatment.

Figure 4 shows the results of the test as in Figure 3 in terms of the amount of soluble p55 TNF-R shed by the cells.

Figure 5 shows the maps of the various p55 TNF-R mutants tested in the study of the structural requirement for the shedding.

Figure 6 shows the results of a test of the ability of PMA to induce shedding of some of the mutants of Figure 5, in terms of the ability of A9 cells expressing them to bind radiolabeled TNF after PMA treatment.

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Figure 7 shows the results of a test of the ability of PMA to induce shedding of some of the mutants of Figure 5, in terms of the amounts of the soluble p55 TNF-R shed by the cells.

Figure 8 shows the results of a test of the ability of PMA and pervanadate to induce shedding of some of the mutants of Figures 2 and 5, in terms of the ability of COS-7 cells expressing them transiently to bind radiolabeled TNF after PMA and pervanadate treatment.

Figure 9 shows the results of a test of the ability of PMA and pervanadate to induce shedding of some of the mutants of Figures 2 and 5, in terms of the amounts of the soluble p55 TNF-R shed by the cells.

In accordance with the present invention, we have now employed a different approach for determining the role of the different domains in the p55 TNF-R on its shedding. For this purpose we replaced different parts of the p55 TNF-R with the corresponding parts of the EGF-receptor, which is not shed in response to agents inducing the shedding of the p55 TNF-R. Study of the shedding of these chimeric molecules indicated that the shedding and its enhancement by inducing agents are not affected by the structure of those regions in the receptor molecule which are embedded in the cell - both the intracellular, as well as the transmembranal domains, nor by that part of the receptor which is shed, namely the cysteine rich module in its extracellular domain. The only region whose structure affects the shedding is the one located close to what appears to be site of cleavage of the receptor, namely - the spacer region in the extracellular domain which links the cysteine rich module to the transmembranal domain. An attempt to define the structural requirements of the shedding further, by detailed study of the mutations in the spacer region on the shedding revealed a rather complicated dependence of the shedding on the sequence on the basis of which it should be possible to design pharmaceutical agents by which the function of the protease can be controlled.

To elucidate the structural requirements of the shedding of the p55-TNF-Rs, we assessed the effects of various mutations of the receptor on its shedding. In a first stage we aimed at a general idea of the relation of the structural requirements for shedding of the receptor and for its signaling. We therefore expressed the various mutants constitutively in mouse A9 cells, which are sensitive to the cytocidal effect of TNF, and then determined their shedding by these cells. However, since the interclonal variation indigenous to this way of proceeding did not allow a sensitive enough assessment of partial effects of mutations on the shedding of the receptors, it seemed preferable to determine the shedding of the receptor mutants by their transient expression in the monkey COS-7 cells. This transient expression assay obviated also the need for the lengthy isolation of cell clones expressing the transfected receptors.

Although the cells applied in the constitutive and transient expression test systems were different, the shedding of the p55 receptor by them occurred in similar manners and was affected similarly by a series of different mutations. In both test systems, phorbol myristate acetate (PMA), an activator of the serine protein kinase C as well as orthovanadate and, to a larger extent, peroxyvanadate, which facilitates tyrosine kinase effects, caused a marked enhancement of the shedding, manifested in increased rate of appearance of soluble receptors in the cells' growth media concomitantly with a decrease in amounts of the cell surface-expressed receptors. This effect was rapid, reaching a significant extent within less than a minute. Its initial rate was little affected by the protein synthesis blocking agent CHI or ammonium chloride, which inhibits lysozomal activities. A significantly decreased rate of shedding was observed also when the cells were incubated at a low temperature. However, in prolonged incubation some shedding could be observed even at 0 °C.

Some shedding of the receptors could be observed also in the absence on any inducing agent. The rate of this spontaneous shedding varied from one receptor mutant to another proportionally to their rates of induced shedding. In the transiently expressing COS-7 cells the rate of spontaneous shedding was rather high, resulting in accumulation of significant amounts of the soluble receptors in the growth media already before application of any inducing agents; it seemed high enough to affect the steady-state level of the cell-surface receptors, as receptors which could be effectively shed were found to be expressed by the COS-7 cells at significantly lower amounts than receptors mutated in a way which decreased their shedding. To account for this difference in expression of the various receptor mutants, we chose to compare the effectivity of shedding of the various mutants by relating to the quantitative ratio of the amount of the soluble receptors which accumulated in the growth media within the shedding induction period and the amount of cell-surface receptors at the start of the induction period.

We have found in accordance with the present invention that shedding of the p55 TNF-R occurs independently of the sequence properties of the intracellular or transmembranal domain of the receptor, or of the structure of that portion of the extracellular domain which is shed. The only region whose amino acid sequence affects the cleavage of the receptor is that in which the cleavage occurs, namely the spacer region which links the cysteine rich module in the extracellular domain with the transmembranal domain. It appears that within this region, not only the residues which are immediately adjacent to the site of cleavage,

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but also some other residues affect this process.

Shedding of the p55 receptor was induced using agents which enhance protein phosphorylation. The involvement of induced phosphorylation in the induction of this process is likely to account, at least partly, for its energy dependence. Apparently, the shedding can be induced through effects of several different kinases, including protein kinase C (Brakebusch, et al., 1992 see above) another, distinct, serine kinase and, as found now, also tyrosine kinases. However, we observed no clear difference in the way by which mutations in the p55 TNF-R affect the shedding induced by PMA, an activator of protein kinase C, or by pervanadate, which facilitates the activity of tyrosine kinases, suggesting that these different kinases activate a common mechanism of shedding.

The protein whose phosphorylation results in the induction of receptor shedding is unlikely to be the receptor itself. The fact that the shedding was not prevented by replacing those domains of the p55 TNF-R which are embedded within the cell, with the corresponding parts of the EGF-R, a receptor which is not shed, seems to rule out an involvement of phosphorylation of the receptor, or of any other induced change in it, in the mechanism of shedding. In that respect, there is particular interest in the fact that, beside lack of an effect on the shedding, those regions in the EGF-R molecule which were introduced into the chimera with the p55 TNF-R do not impose receptor uptake, which seems to exclude a role for induced uptake in the shedding. The resistance of the shedding to ammonium chloride and chloroquine, agents known to inhibit degradative processes within intracellular acidic compartments and the fact that some shedding occurred even when incubating the cells at 0 °C, which should prevent any uptake of proteins, exclude further a role of receptor uptake in the process.

The sequence requirements for the shedding of the p55 TNF-R in the mouse A9 cells and in the monkey COS-7 cells are very similar, perhaps identical, suggesting that the same or similar protease(s) take part in the shedding in these different cells. More specifically, the findings in accordance with the present invention indicate that a short amino acid sequence in the p55 TNF-R is essential and sufficient for its shedding. This sequence is in the so-called spacer sequence between the transmembranal region and the Cys-rich extracellular domain region of the receptor, with the amino acid residues Asn 172, Val 173, Lys 174 and Gly 175, in particular the Val 173, being most important. Interestingly, the shedding of the receptor is generally independent of the side chain identity of the above noted residues, with the exception of a limited dependence on the identity of Val 173 (e.g. replacement of Asn 172, Lys 174 and Gly 175 by Ala) did not adversely effect the shedding of the receptor. However, mutations which change the conformation of the protein (e.g. replacement of any of the above residues with Pro) adversely effected the shedding process. This sequence requirement is quite different from any sequence requirement for the function of known proteases so far described.

Identification and purification of the protease, despite lack of detailed knowledge about its specific biochemical properties can be effected by, e.g. affinity chromatography. For this purpose the constructs shown to act as protease inhibitors are coupled to a conventional affinity chromatography column, i.e. Affi-Gel 10. Other known solid supports such as other agaroses, resins or plastics may be employed.

A variety of biological materials are available as sources of protease activity. These include tissues, cells, or extracts, or fluids associated therewith which preferably, but not necessarily, are of immunological origin. Established cell lines can also be utilized. In general, any cell expressing TNF-Rs can be employed as the source for the protease.

Cells may be used as is in the affinity purification, or may be stimulated to produce higher levels of protease using known activators for the particular cells employed.

A protease according to the invention, or any molecule derived therefrom which augments the activity of the protease may be employed to decrease the amount of cell-bound TNF-Rs and thus protect from over-response to TNF. Thus the proteases according to the invention are indicated for the treatment of diseases caused by an excess of TNF, either administered exogenously, or produced endogenously.

Conversely, the protease inhibitors can be used to prevent shedding of the TNF-Rs, e.g. in cases where the beneficial activities of TNF are to be enhanced, e.g. in the treatment of tumor cells by TNF. This will lead to an increase of the effectivity of the antitumor activity.

The invention will now be illustrated by the following non-limiting examples:

General Procedures and Materials

a) **Construction of p55-TNF-R mutants and p55-TNF-R - EGF-R chimeras**

The cDNA of the hu-p55-TNF-R (Nophar, Y. et al., EMBO J., **Vol. 9**, pp.3269-3278 (1990)) was digested with BanII and NheI, resulting in removal of most of the 5' and 3' non-coding sequences. The p55-TNF-R mutants were generated by oligonucleotide-directed mutation, using the "Altered Sites" mutagenesis kit

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(Promega, Madison, WI). The mutations were confirmed by sequencing the regions of interest. Fragments of the hu-p55-TNF-R and of the EGF receptor (EGF-R) cDNAs used for creation of receptor chimeras were produced by PCR, using the 'Vent' DNA polymerase (New England Biolabs, Beverly, MA). Some of the chimeras (designated C_3, C_4, C_5 and C_6 in Fig. 2), were constructed using mouse EGF-R cDNA (Avivi A., et al., Oncogene, **Vol. 6**, pp. 673-676 (1991)), kindly provided by Dr. D. Givol of the Weizmann Institute, Rehovot, Israel, and others (e.g. that designated C_9 in Fig. 2) were constructed using human EGF-R cDNA (Merlino G.T., et al., Molec. Cell. Biol., **Vol. 5**, pp. 1722-1734 (1985)), kindly provided by Drs. G. Merlino and I. Pastan, NIH, Bethesda, MD. For constitutive expression of the wild-type or mutated receptors in A9 cells, they were introduced into the eukaryotic expression vector pMPSVEH (Artelt P. et al., Gene, **Vol. 68**, pp. 213-219 (1988)), kindly provided by Dr. H. Hauser, GBF, Braunschweig, Germany) which contains the myeloproliferative sarcoma virus promoter. For transient expression of the receptors in COS-7 cells, they were introduced into the pEXV1 vector (Miller, J. and Germain, R.N., J. Exp. Med., Vol. 164, pp. 1478-1489 (1986)), which contains the SV40 virus enhancer and early promoter. In all of the hu-p55-TNF-R constructs expressed in COS-7 cells, the receptor was cytoplasmically truncated from residue 207 downstream (in addition to the other specified mutations).

b) **Constitutive and transient expression of the wild type and mutant receptors**

A9 (Littlefield, J.W., Nature, **Vol. 203**, pp. 1142-1144 (1964)) and COS-7 (Gluzman, Y., Cell, **Vol. 23**, pp. 175-182 (1981)) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum (FCS), 100 u/ml penicillin and 100 µg/ml streptomycin (growth medium). The A9 cells were transfected with pMPSVEH expression constructs together with the pSV2neo plasmid, and cell colonies constitutively expressing these constructs were isolated as previously described (Brakebusch, C., et al., EMBO J., **Vol. 11**, pp. 943-950 (1992)). Transient expression of pEXV1 constructs in COS-7 cells was carried out as follows: one day after the COS-7 cells were seeded at 60% cell density they were transfected by applying the DNA of the constructs to them for 4 h. at a concentration of 3 µg/ml in DMEM (4ml/10cm dish, 10ml/15cm dish) containing DEAE dextran (200 µg/ml, Pharmacia, Uppsala, Sweden). The cells were then rinsed with DMEM and incubated for 2 min in PBS (0.154 M sodium chloride plus 10 mM sodium phosphate, pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10% (v/v) DMSO. The cells were rinsed and further incubated for 10 h. in growth medium, then detached by trypsinization and seeded either into 1,5 cm culture plates (10^5 cells/plate) or (to assess the shedding of metabolically labeled EGF-R) into 15 cm plates ($1.2X10^6$ cells/plate). Expression and efficacy of shedding of receptors encoded by the transfected constructs were assessed 48 h. later.

c) **Determination of binding of TNF and EGF to cells**

Recombinant human TNF-α (TNF, $6X10^7$ U/mg of protein, Genentech Co., San Francisco, CA, kindly provided by Dr. G. Adolf of the Boehringer Institute, Vienna, Austria) was radiolabelled with chloramine T to a specific radioactivity of 500 Ci/mmol (Israel, S., et al., Immunol. Lett., **Vol. 12**, pp. 217-224 (1986)). EGF (β-urogastrone, Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) was labeled with [^{125}I] to a specific radioactivity of 300 Ci/mmol, using the IODOGEN reagent (Pierce Chemical Co., Rockford, IL, USA), following the instructions of the manufacturer. Binding of radiolabelled TNF and EGF to cells was determined by applying them to the cells on ice at a concentration of 1 nM, either alone or with a 100-fold excess of unlabelled cytokines, as described previously (Brakebusch et al., (1992) *supra*).

d) **Measurement of the shedding of the soluble forms of hu-p55-TNF-R and EGF-R**

A9 cells constitutively expressing the transfected constructs were seeded, 24 hrs prior to the assay, into 1.5 cm tissue culture plates at a density of $2.5X10^5$ cells/plate. COS-7 cells expressing transiently transfected constructs were seeded into 1.5 cm tissue culture plates, 48 hrs prior to the assay, as described above. At time zero, some of the plates were placed on ice to determine the binding of radiolabelled TNF or EGF to the cells prior to induction of shedding. The medium in the other plates was replaced with fresh DMEM (200µl/plate) either without serum (for tests in which PV was the agent used to induce shedding) or with 10% FCS (for the other tests). Unless otherwise indicated, PMA (20ng/ml) or PV (100µM, prepared as described in Fantus, I. G., et al., Biochemistry, **Vol. 28**, pp. 8864-8871 (1989)), was applied to the cells for 1 hr. Application of Chloroquine (50µg/ml), ammonium chloride (10 mM) or cycloheximide (50µg/ml) to the cells was done 30 min prior to application of PMA or PV, followed by further incubated with these agents for 20 min after addition of the latter reagents. Upon termination of incubation with the shedding-inducing agents, the plates were transferred to ice to determine the binding of radiolabelled TNF or EGF to the cells. The amounts of the soluble form of the hu-p55-TNF-R in the cells' growth media were determined after centrifugation at 3000g for 5 min to remove detached cells and cell debris, followed by 5-fold concentration of the media, using the SpeedVac concentrator (Savant, Farmingdale, NY). The determination was performed by two-site capture ELISA, using a mouse

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monoclonal antibody and rabbit antiserum against this protein, as described (Aderka, D. et al., Cancer Res., Vol. 51, pp. 5602-5607 (1991)).

To assess the formation of the soluble form of the EGF-R, COS-7 cells transfected with the EGF-R constructs ($1.2X10^6$ cells, seeded into 15cm dishes as described above) were labelled with [^{35}S] methionine by incubation for 10 hrs at 37°C in DMEM (methionine-free) containing 70µCi/ml [^{35}S] methionine and 2% dialyzed FCS. The cells were then rinsed and further incubated for 1 hr in growth medium containing PMA (20ng/ml). The medium was collected, cleared of cell debris by spinning, and then further cleared of proteins that bind nonspecifically to protein A by incubating it twice at 4°C for 4 hrs with immobilized protein A (Repligen Inc., Cambridge, MA; 100µl/7ml medium/plate), once alone and once in the presence of 10µg irrelevant mouse monoclonal antibodies. Immunoprecipitation was then performed by incubation of the medium samples at 4 °C for 2.5 hrs with a monoclonal antibody against the human EGF-R, or, as a control, with a monoclonal antibody against the h-p55-TNF-R, each at 5µg/sample, followed by further incubation for 2.5 hrs with immobilized protein A (40µl). The protein A beads were washed three times with PBS containing 0.2% sodium deoxycholate and 0.2% NP-40, and the proteins bound to them were then analyzed by SDS-PAGE under reducing conditions (7.5% acrylamide). Autoradiography was performed after treatment of the gel with the Amplify intensifying reagent (Amersham International plc, Amersham, UK).

e) **Presentation of the data**

All data on receptor shedding presented in the following Examples 1-3 and their accompanying figures, Figs. 3, 4 and 6-9, are representative examples of at least four experiments with qualitatively similar results, in which each construct was tested in triplicate. It should be noted that the efficacy of construct expression varied rather extensively (in their constitutive expression among different cell clones, and in their transient expression among different constructs). The data on the extent of shedding have therefore been normalized by relating them to the initial receptor levels in the cells, prior to the induction of shedding. The amounts of cell-surface bound receptors obtained after induction are presented as percentages of their initial amounts (see Figs. 3, 6 and 8) and hence those forms of the receptors which are shed show a lower percentage of remaining cell-surface receptors than do those forms which are not shed. The amounts of soluble receptors formed as a consequence of the shedding are presented in relative units (see Figs. 4, 7 and 9), i.e. amount of soluble receptors produced during shedding induction (in pgs) per amount of cells-surface receptors just prior to shedding induction (in cpm X 10^{-3} of cell bound radiolabelled TNF, and hence those forms of receptors which are shed show higher amounts than those which are not shed. Residue numbering in the h-p55-TNF-R is according to Schall, T.J. et al., Cell, **Vol. 61**, pp. 361-370 (1990), in the mouse EFG-R according to Avivi, A. et al., (1991) *supra*, and in the human EGF-R, according to Ulrich, A. et al., Nature, **Vol. 309**, pp. 418-425 (1984).

Example 1: Use of chimeras of the p55 TNF receptor and the EGF-R for assessing the role of different regions in the TNF receptor in its shedding.

Our study of previous work concerning the shedding of cytoplasmic deletion mutants of the p55 receptor indicated that the shedding, and its enhancement by PMA, occur independently of the intracellular domain of the receptor. To explore further the role of the different domains in the receptor in its shedding, we now attempted to replace them with the corresponding region in a receptor which is not shed. The receptor for the epidermal growth factor (EGF) seemed suitable for that purpose. PMA induces a decrease in expression of this receptor, yet apparently not by its shedding but by induction of uptake of this receptor into the cell. This uptake was related to induced phosphorylation of its intracellular domain. A series of chimeras (see Fig. 2 for various chimeras designated C_3, C_4, C_5, C_6 and C_9) between the p55 TNF-R and a cytoplasmic deletion mutant of the EGF-R was created and tested for the extent of shedding of chimeras in response to PMA and pervanadate (see General Procedures above). The mutated EGF-R was not shed, nor taken up by cells in response to PMA, nor in response to pervanadate (results not shown). Both agents did induce the shedding of a chimeric receptor comprised of the remaining part of the intracellular and the transmembranal domain of the EGF receptor, and the extracellular domain of the p55 TNF receptor (Fig. 2, chimeras C_5 and C_6). However, chimeric receptors in which the "spacer" region in the extracellular domain of the p55 TNF-R, which links the cysteine rich module with the transmembranal domain was deleted (Fig. 2, chimeras C_3 and C_4), or replaced with the corresponding region in the EGF-R (Fig. 2, chimera C_9), could not be shed. These findings indicated that the structural requirement for the shedding of the p55 TNF receptor and for its enhancement by PMA and pervanadate are fully confined to the spacer region.

As shown in Figures 3 and 4, which present the results of a test of the shedding of p55 TNF/EGF receptor chimeras shown in Figure 2 expressed constitutively in A9 cells, chimeras which contain the

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spacer region of the p55 TNF-R (chimeras C_5 and C_6) are shed while those that do not (chimeras C_3 and C_4), are not shed in response to PMA. As shown in Figures 8 and 9, the same hold true for the shedding of these constructs in response to either PMA of pervanadate (PV) by COS-7 cells which express them transiently.

Example 2: Effects of amino acid deletions in the spacer region on the shedding

We have previously found that p55 TNF-R mutants from which most of the spacer region was deleted do not shed spontaneously or in response to PMA (Brakebusch D., et al., Tumor Necrosis-Factor IV (Ed. W. Fiers) S. Karger, Verlag (Basel) pp191-198 (1993)). In order to further define those amino acid residues whose deletion accounted for the lack of shedding of the receptors, we created receptor mutant forms in which various couples of consecutive amino acids within the spacer regions were deleted, and examined their shedding (see General Procedures above). The various deletion mutants studied are presented in the upper part of Fig. 5, where the symbol "Δ" denotes the deletion and the numeral(s) following the Δ denote the amino acid residue(s) which have been deleted.

As shown in Figures 8 and 9, any deletion of two or more amino acids within the spacer region results in some decrease in effectivity of the shedding of the receptor from transiently expressing COS-7 cells in response to PMA or pervanadate. However, the most dramatic decrease in the effectivity occurs in deletion of Val 173 or the couple 173-174. A somewhat less effective decrease was observed in deleting couples 172-173 and 174-175. The data in Figures 6 and 7, as to the shedding from cells which constitutively express the receptor mutants show that deletions 172-173 and 173-174 have also dramatic reducing effect on the shedding to the receptor form A9 cells which express them constitutively. These data show that residues 173, 174 and 175 have an important role in determining the specificity of the protease which cleaves the p55 TNF-R. Besides, they imply that also other structural constraints in the spacer region affect its shedding.

Example 3: Effects of amino acid replacements in the spacer region on the shedding

To further define those amino acid residues within the spacer region which affect receptor shedding, we replaced them, one by one, with alanine, (for the alanine replacement mutants, see the mid-section of Fig. 5, where the normally occurring amino acid residue is denoted at the left of the numeral(s) and the alanine (A) replacing that residue is denoted at the right of the numeral, the numeral being the position of the replacement). Assessing the shedding of these mutated receptors, after expressing them constitutively in A9 cells, or transiently in COS-7 cells, failed to reveal an effect of any of these mutations, as is shown in figs. 6 and 7 (A9 cells) and in Figs. 8 and 9 (COS-7 cells), wherein the alanine replacement mutants are denoted by an "A" before the residue number of the replacement.

However replacement of residues 173, 174 and 175 with proline (P) resulted in a drastic decrease of the shedding of the receptors by A9 cells which express them transiently. Moreover, certain other amino acid replacements at the 173 site also resulted in a significant decrease in the effectivity of shedding (Figures 6-9). These data implied again that residues 173-175 have an important role in restricting the function of the protease which cleaves the p55 TNF-R (for the proline (P) and other amino acid replacement mutants, see the lower section of Fig. 5, where the denotations of the mutations are as above).

Example 4: Affinity purification of the protease

A peptide whose structure corresponds to that of the spacer region of the TNF-R mutated in such a way that it interferes with its cleavage, yet not with its recognition by the protease, is linked covalently to the resin on an affinity purification column. Detergent extracts of membranes isolated from cells which express the protease capable of cleaving the p55 TNF-R are passed through the column and the unbound material is washed. Thereafter the protease is eluted, either by increasing the salt concentration or by decreasing the pH, and further purified.

Example 5: Antibodies to the protease

Female Balb/C mice (8 weeks old) are injected with 1 μg protease obtained in Example 4 in an emulsion of complete Freund's adjuvant into the hind foot pads, and about three weeks later, subcutaneously into the back in incomplete Freund's adjuvant. The other injections are given at weekly intervals, subcutaneously in PBS. Final boosts are given 4 days (i.p.) and 3 days (i.v.) before the fusion in

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PBS. Fusion is performed using NSO/Mr cells and lymphocytes prepared from both the spleen and the local lymphocytes of the hind legs as fusion partners. The hybridomas are selected in DMEM supplemented with HAT, 15% horse serum and gentamycin 2 µg/ml. Hybridomas that are found to produce antibodies to the protease are subcloned by the limited dilution method and injected into Balb/C mice that were primed with pristane for the production of ascites. Immunoglobulins are isolated from the ascites by ammonium sulfate precipitation (50% saturation) and then dialyzed against PBS containing 0.02% azide. Purity is estimated by analysis on SDS-PAGE and staining with Commassie blue. The isotypes of the antibodies are defined with the use of a commercially available ELISA kit (Amersham, U.K.).

Example 6: Affinity purification

Antibodies against the protease can be utilized for the purification of the protease by affinity chromatography, according to the following procedure. The monoclonal antibodies for affinity chromatography are selected by testing their binding capacity for the radiolabeled antigen in a solid phase radio immunoassay. Ascites from all hybridomas are purified by ammonium sulfate precipitation at 50% saturation followed by extensive dialysis against PBS. PVC 96-well plates are coated with the purified McAbs, and after blocking the plates with PBS containing 0.5% BSA, 0.05% Tween 20 (Sigma) and 0.02% NaN_3, the wells are incubated with 50,000 cpm ^{125}I-TNF for 2 h at 37 °C, then washed and the radioactivity which binds to each well is quantitated in the gamma-counter. The antibodies with the highest binding capacity are examined for their performance in immunoaffinity chromatography.

Polyacryl hydrazide agarose is used as resin to immobilize the antibodies. The semipurified immunoglobulins are concentrated and coupled as specified by Wilchek and Miron (Methods in Enzymology, **Vol 34**, pp.72-76 (1979)). Antibody columns of 1 ml bed volume are constructed. Before use, all columns are subjected to 10 washes with the elution buffer, each wash followed by neutralization with PBS. The columns are loaded with the protease obtained in Example 4 in PBS with 0.02% NaN_3. The flow rate of the columns is adjusted to 0.2 to 0.3 ml per minute. After loading, the columns are washed with 50 ml PBS and then eluted with a solution containing 50 mM citric acid, pH 2.5, 100 mM NaCl and 0.02% NaN_3. Fractions of 1 ml are collected. Samples of the applied protease, the last portion of the wash (1 ml) and of each elution fraction (8 fractions of 1 ml per column) are taken and tested for protein concentration. All protein measurements are effected according to a microflurescamin method in comparison to a standard solution containing 100 µg BSA/ml (Stein, S. and Moschera, J., Methods Enzymol., **Vol. 79**, pp.7-16 (1981)).

Example 7: Chromatographic purification of the protease

Crude preparations of the protease, obtained by detergent extraction of membranes of cells which express the protease, or partially purified preparations of the protease formed in Example 4 are subjected to a series of chromatographic fractionation steps e.g., based on charge, size, isoelectric point or hydrophobicity of the fractionated proteins. Throughout the fractionation steps the protease activity is followed by determining the ability of the tested fraction to cause cleavage of the p55 TNF-R or of a peptide derived from it at the same site and by the same sequence requirements as those found for the cleavage of the receptor in cells.

Example 8: Cloning of the protease

Cells exhibiting the protease activity (namely - exhibiting inducible shedding of their p55 TNF-R) are mutated by chemical mutagens. Cell mutants deficient in the protease activity are isolated by FACS staining for the p55 TNF-R after induction of the shedding. The sequence of the nucleotides in the p55 TNF-R gene within the mutated cells is examined to confirm that the inability to shed in indeed due to aberration of the cleavage mechanism and not to a mutation in the spacer region in the receptor. The mutant cells are then transfected either with the genomic DNA or with a cDNA library derived from cells which express the protease. Clones of cells which have regained the ability to shed due to the transfection are isolated by FACS analysis as above and the transfected gene which has complemented their defect is isolated. In some of these mutants the transfected gene or cDNA that has complemented the defect is expected to be the gene for the protease.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Yeda Research and Development Company, Ltd.
(B) STREET: P.O. Box 95
(C) CITY: Rehovot
(E) COUNTRY: Israel
(F) POSTAL CODE (ZIP): 76100

(ii) TITLE OF INVENTION: Molecules influencing the shedding of the TNF receptor, their preparation and their use

(iii) NUMBER OF SEQUENCES: 44

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 94 11 6018.6

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IL 107268
(B) FILING DATE: 12-OCT-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2175 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:256..1620

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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                   1               5                  10

CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT      339
Leu Val Leu Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile
          15                  20                  25

GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT      387
Gly Leu Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys
      30                  35                  40

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Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
 45                  50                  55                  60

AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG      483
Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly
                 65                  70                  75

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Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser
             80                  85                  90

GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA      579
Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu
         95                  100                 105

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    110                 115                 120

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Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu
125                 130                 135                 140

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Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu
                145                 150                 155

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Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe
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Phe Leu Arg Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser
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CAA CGG TGG AAG TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA       1011
Gln Arg Trp Lys Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr
            240                 245                 250

CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT AAG CCC CTG GCC       1059
Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala
        255                 260                 265

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Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp
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Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro
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365                 370                 375                 380

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CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG CGG CGC ACG CCG       1491
Arg Glu Ala Gln Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro
            400                 405                 410

CGG CGC GAG GCC ACG CTG GAG CTG CTG GGA CGC GTG CTC CGC GAC ATG       1539
Arg Arg Glu Ala Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met
        415                 420                 425

GAC CTG CTG GGC TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC       1587
Asp Leu Leu Gly Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro
    430                 435                 440

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GAGCTGTGGA CTTTTGTACA TACACTAAAA TTCTGAAGTT AAAAAAAAAA AAAAA 2175

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 455 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
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20 25 30

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35 40 45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
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Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
85 90 95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
115 120 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
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Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
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385 390 395 400

Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala
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Pro Ala Pro Ser Leu Leu Arg
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Pro Ser Ile Ala Thr
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Leu Cys Leu Pro Ser Phe Ala Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
1 5 10 15

Gly Thr Ser Phe Glu Val Trp Pro Ser Gly Pro Lys Ile Pro Ser Ile
20 25 30

BNSDOCID: <EP___0657536A1_I_>

Ala Thr

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
1 5 10 15

Gly Thr Ser Phe Ala Thr
20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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1 5 10 15

Leu Gln Gly Cys Glu Val Trp Pro Ser Gly Pro Lys Ile Pro Ser Ile
20 25 30

Ala Thr

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

BNSDOCID: <EP___0657536A1_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
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Gly Thr Thr Val Leu Leu
20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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(2) INFORMATION FOR SEQ ID NO: 10:

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(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

BNSDOCID: <EP___0657536A1_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ile Glu Asn Val Lys Gly Thr Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Ile Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
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(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Ile Glu Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

BNSDOCID: <EP___0657536A1_I_>

(2) INFORMATION FOR SEQ ID NO: 15:

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(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Ile Glu Asn Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

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(2) INFORMATION FOR SEQ ID NO: 17:

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

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(2) INFORMATION FOR SEQ ID NO: 18:

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

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1 5 10

(2) INFORMATION FOR SEQ ID NO: 19:

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

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1 5 10

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

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1 5 10

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid

BNSDOCID: <EP___0657536A1_I_>

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Ile Glu Asn Val Lys Gly Thr Glu Asp Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Ile Glu Asn Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Ile Ala Asn Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

BNSDOCID: <EP___0657536A1_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Ile Glu Ala Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Ile Glu Asn Ala Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Ile Glu Ala Ala Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

BNSDOCID: <EP___0657536A1_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Ile Glu Asn Val Ala Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Ile Glu Asn Val Lys Ala Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Ile Glu Pro Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Ile Glu Asn Pro Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

BNSDOCID: <EP___0657536A1_I_>

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Ile Glu Asn Val Pro Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Ile Glu Asn Val Lys Pro Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Ile Glu Asn Val Lys Pro Pro Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid

BNSDOCID: <EP___0657536A1_I_>

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Ile Glu Asn Val Lys Pro Thr Pro Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Ile Glu Glu Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Ile Glu Asp Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

BNSDOCID: <EP___0657536A1_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Ile Glu His Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Ile Glu Ile Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Ile Glu Asn Asp Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

BNSDOCID: <EP___0657536A1_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Ile Glu Asn Gly Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Ile Glu Asn Arg Lys Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Ile Glu Asn Val Glu Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

Ile Glu Asn Val Gln Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

BNSDOCID: <EP___0657536A1_I_>

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

Ile Glu Asn Val Thr Gly Thr Glu Asp Ser Gly Thr Thr Val
1 5 10

Claims

1. A protease capable of cleaving the soluble TNF-R from the cell-bound TNF-R.

2. A protease according to claim 1, wherein the TNF-R is the p55 TNF-R.

3. A method for preparing a protease according to claim 1 or claim 2, comprising
   a) preparing a construct comprising an amino acid sequence inhibiting the protease,
   b) affixing said construct to an affinity chromatography column,
   c) passing a biological sample containing the protease through the column, and
   d) recovering the protease from the column.

4. A DNA molecule comprising a nucleotide sequence coding for the protease according to claim 1 or claim 2.

5. A replicable expression vehicle comprising the DNA molecule of claim 4 and capable, in a transformant host cell, of expressing the protease defined in claims 1 and 2.

6. A host cell selected from a prokaryotic and eukaryotic cell transformed with the replicable expression vehicle of claim 5.

7. A process for producing a protease capable of cleaving a TNF-R comprising the steps of: (a) culturing transformant host cell according to claim 6 in a suitable culture medium, and (b) isolating said protease.

8. A pharmaceutical composition comprising a protease capable of cleaving TNF-Rs as active ingredient together with a pharmaceutically acceptable carrier.

9. A protease capable of cleaving TNF-Rs for use in antagonizing the deleterious effect of TNF in mammals in the treatment of conditions wherein excess of TNF is formed endogenously or is exogenously administered.

10. An inhibitor to a protease capable of cleaving TNF-Rs for use in maintaining prolonged beneficial effects of TNF in mammals, when used in conjunction with TNF exogenously administered.

11. An antibody to the protease according to claim 1 or claim 2, capable of binding to the protease and either neutralizing the enzymatic activity of the protease or preventing the protease from binding to the receptor.

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12. An antibody according to claim 11, being a polyclonal antibody.

13. An antibody according to claim 11, being a monoclonal antibody.

14. An antibody according to claims 12 or 13, being a murine antibody.

15. An antibody according to claim 12 or 13, being a human antibody.

16. A pharmaceutical composition containing a protease according to claim 1 or 2 optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

17. The pharmaceutical composition of claim 16 for enhancing soluble TNF-R function.

18. A pharmaceutical composition containing an antibody according to any one of claims 11 to 15, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

19. The pharmaceutical composition of claim 18 for enhancing TNF function.

20. An inhibitor of a protease according to claim 1 or 2 comprising any one of the following constructs depicted in Figure 5:
    a)Δ 172-173
    b)Δ 173-174
    c)Δ 174-175
    d)Δ 173
    e)V 173 P
    f)K 174 P
    g)G 175 P
    h)V 173 D
    i)V 173 G

21. An inhibitor according to claim 20, comprising muteins of constructs e) to i).

22. A protease inhibitor comprising a peptide having the amino acid sequence Asn-172 to Thr-182.

23. A gene encoding the protease whose cloning procedure involves the use of the information on the structural requirements characterizing the shedding of the p55 TNF-R by cells.

24. A protease whose purification involves the use of the information on the structural requirements characterizing the shedding of the p55 TNF-R by cells.

BNSDOCID: <EP___0657536A1_I_>

Figure 1

BNSDOCID: <EP___0657536A1_I_>

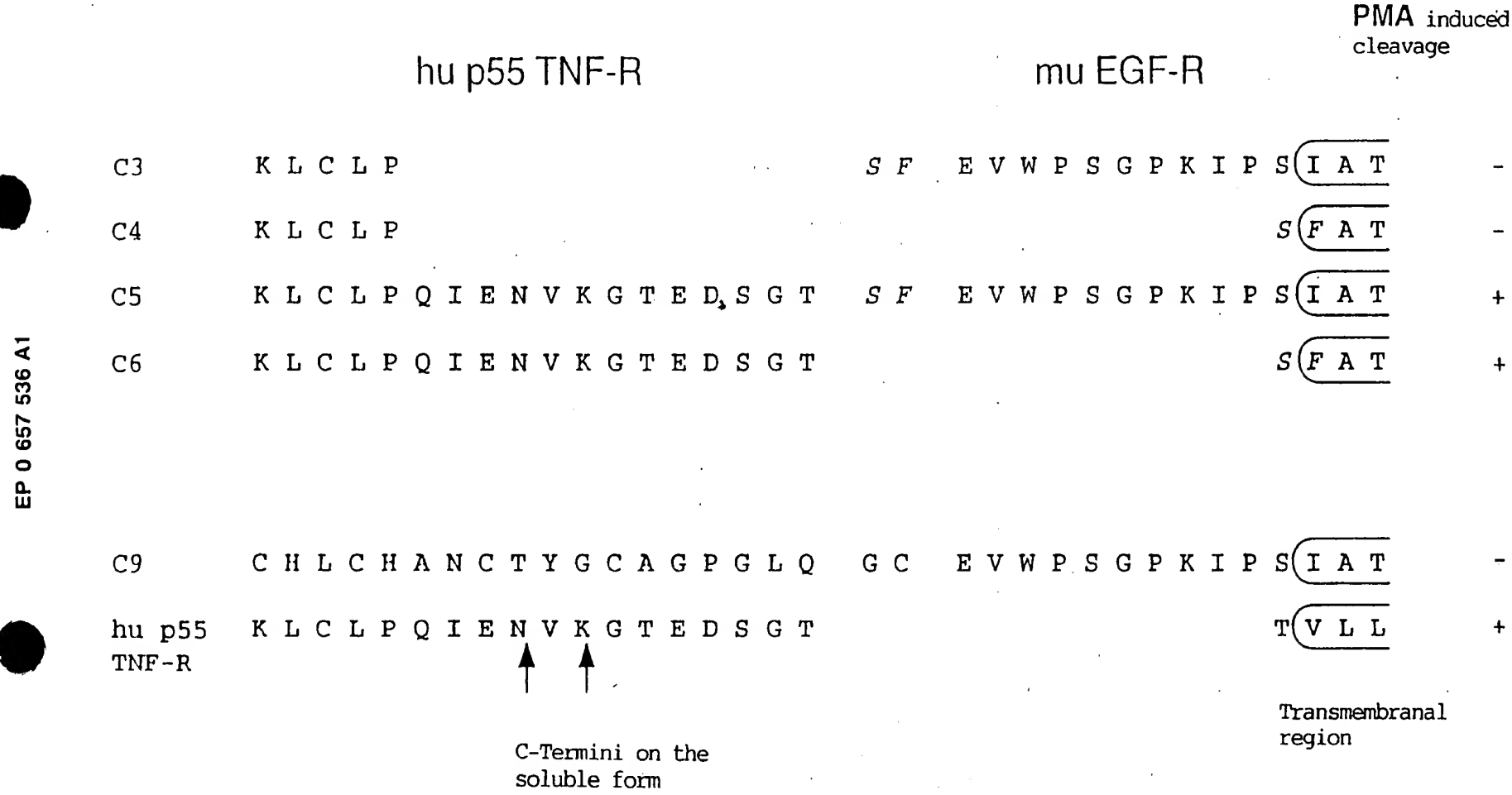

Figure 2

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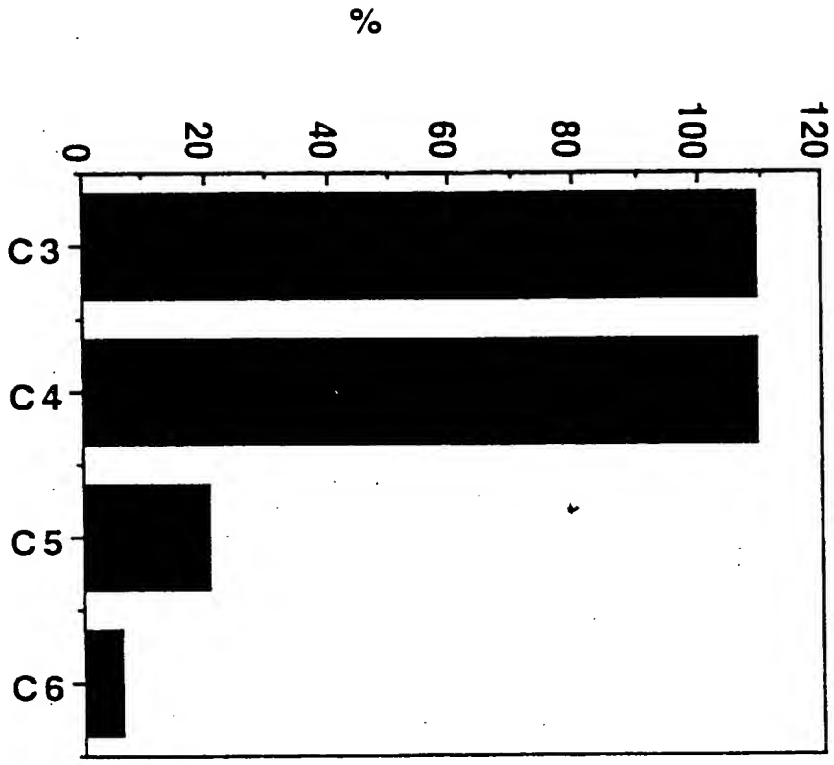

Figure 3

BNSDOCID: <EP___0657536A1_I_>

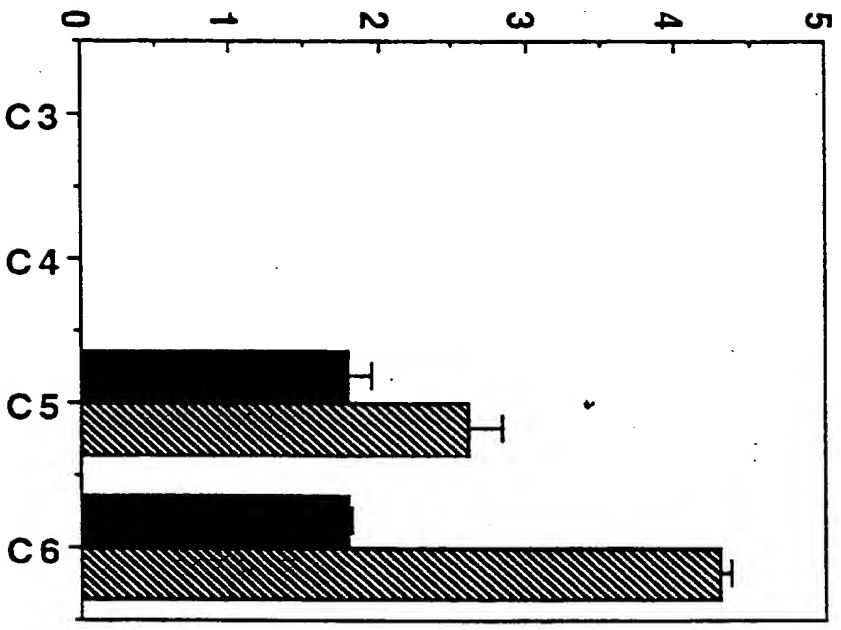

Figure 4

BNSDOCID: <EP___0657536A1_I_>

I	E	N	V	K	G	T	E	D	S	G	T	T	V	Wild type
										G	T	T	V	Δ 170-179
					G	T	E	D	S	G	T	T	V	Δ 170-174
I	E	N	V	K						G	T	T	V	Δ 175-179
I			V	K	G	T	E	D	S	G	T	T	V	Δ 171-172
I	E			K	G	T	E	D	S	G	T	T	V	Δ 172-173
I	E	N			G	T	E	D	S	G	T	T	V	Δ 173-174
I	E	N	V			T	E	D	S	G	T	T	V	Δ 174-175
I	E	N	V	K			E	D	S	G	T	T	V	Δ 175-176
I	E	N	V	K	G			D	S	G	T	T	V	Δ 176-177
I	E	N	V	K	G	T			S	G	T	T	V	Δ 177-178
I	E	N	V	K	G	T	E			G	T	T	V	Δ 178-179
I	E	N	V	K	G	T	E	D			T	T	V	Δ 179-180
I	E	N		K	G	T	E	D	S	G	T	T	V	Δ 173
I	A	N	V	K	G	T	E	D	S	G	T	T	V	E 171 A
I	E	A	V	K	G	T	E	D	S	G	T	T	V	N 172 A
I	E	N	A	K	G	T	E	D	S	G	T	T	V	V 173 A
I	E	A	A	K	G	T	E	D	S	G	T	T	V	NV 172-173 AA
I	E	N	V	A	G	T	E	D	S	G	T	T	V	K 174 A
I	E	N	V	K	A	T	E	D	S	G	T	T	V	G 175 A
I	E	P	V	K	G	T	E	D	S	G	T	T	V	N 172 P
I	E	N	P	K	G	T	E	D	S	G	T	T	V	V 173 P
I	E	N	V	P	G	T	E	D	S	G	T	T	V	K 174 P
I	E	N	V	K	P	T	E	D	S	G	T	T	V	G 175 P
I	E	N	V	K	P	P	E	D	S	G	T	T	V	T 176 P
I	E	N	V	K	P	T	P	D	S	G	T	T	V	E 177 P
I	E	E	V	K	G	T	E	D	S	G	T	T	V	N 172 E
I	E	D	V	K	G	T	E	D	S	G	T	T	V	N 172 D
I	E	H	V	K	G	T	E	D	S	G	T	T	V	N 172 H
I	E	I	V	K	G	T	E	D	S	G	T	T	V	N 172 I
I	E	N	D	K	G	T	E	D	S	G	T	T	V	V 173 D
I	E	N	G	K	G	T	E	D	S	G	T	T	V	V 173 G
I	E	N	R	K	G	T	E	D	S	G	T	T	V	V 173 R
I	E	N	V	E	G	T	E	D	S	G	T	T	V	K 174 E
I	E	N	V	Q	G	T	E	D	S	G	T	T	V	K 174 Q
I	E	N	V	T	G	T	E	D	S	G	T	T	V	K 174 T

Figure 5

BNSDOCID: <EP___0657536A1_I_>

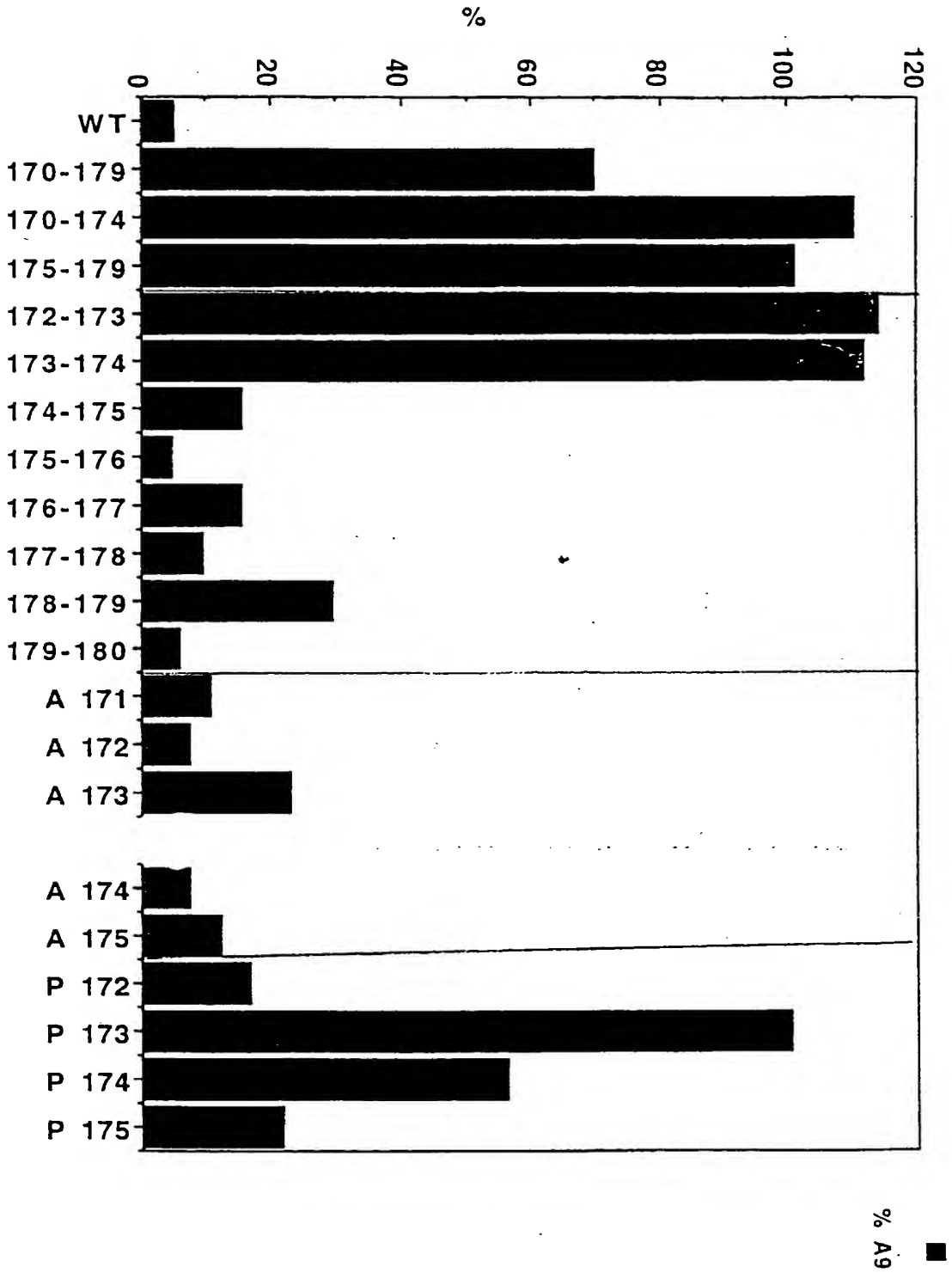

Figure 6

BNSDOCID: <EP___0657536A1_I_>

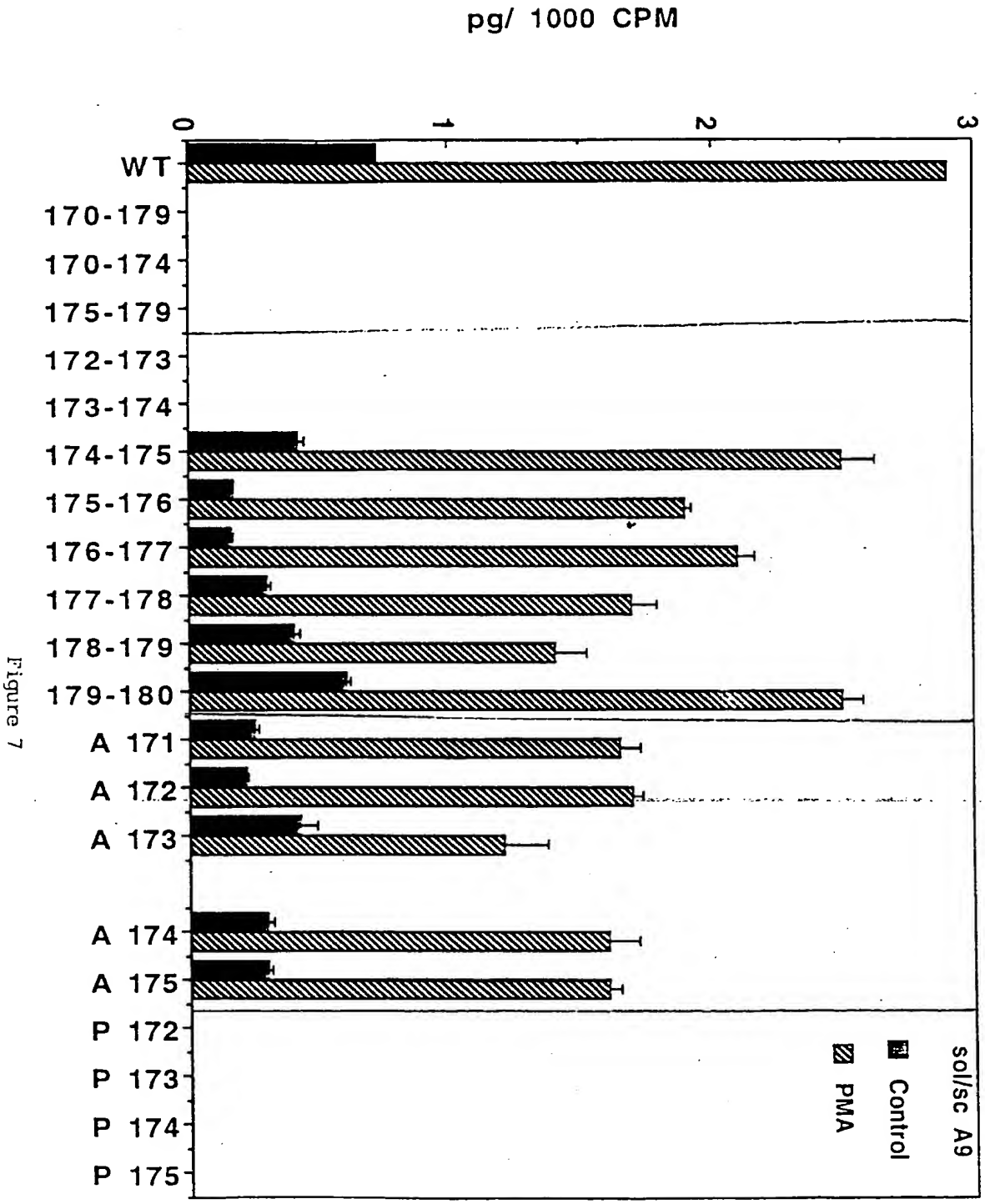

Figure 7

BNSDOCID: <EP___0657536A1_I_>

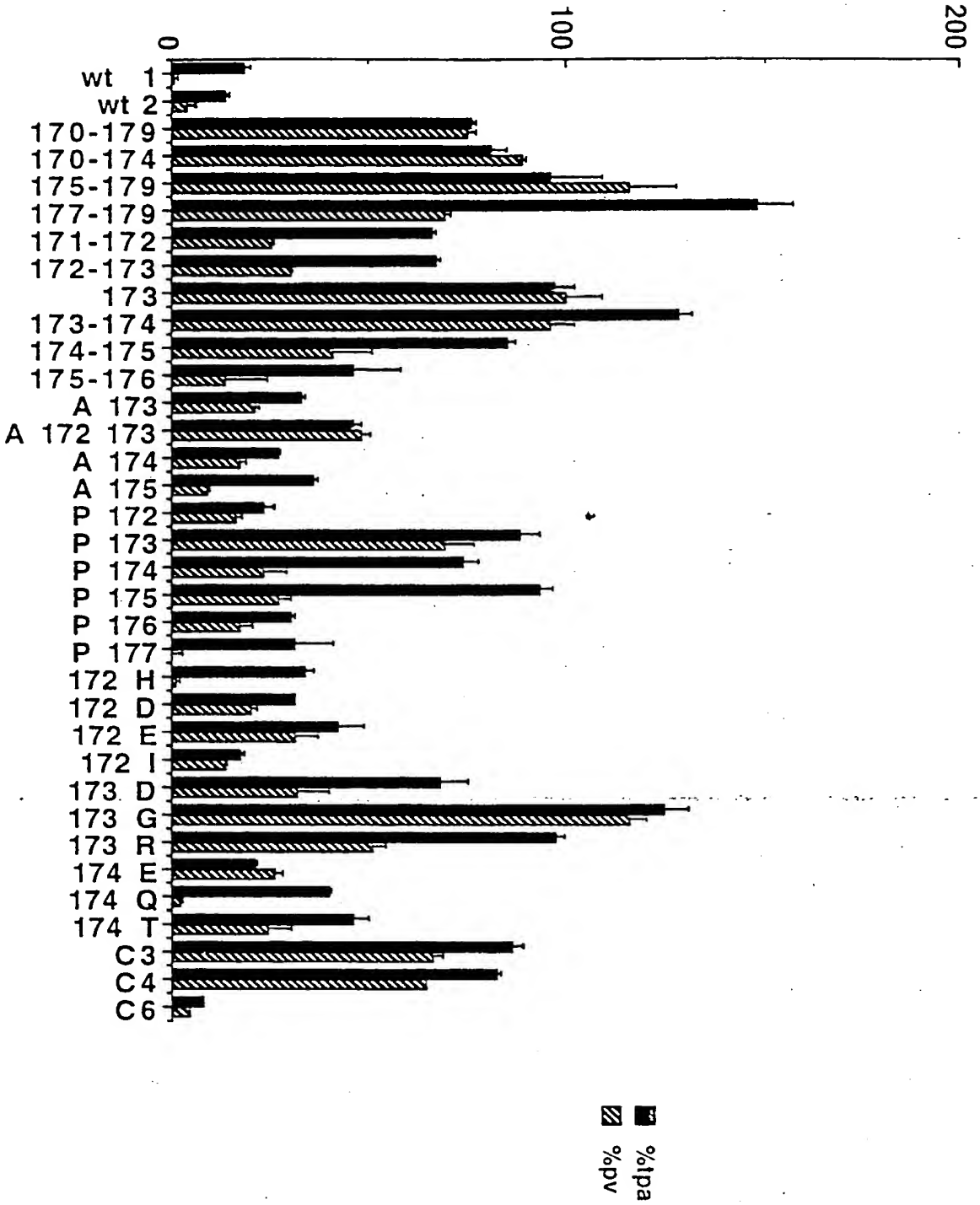

Figure 8

BNSDOCID: <EP___0657536A1_I_>

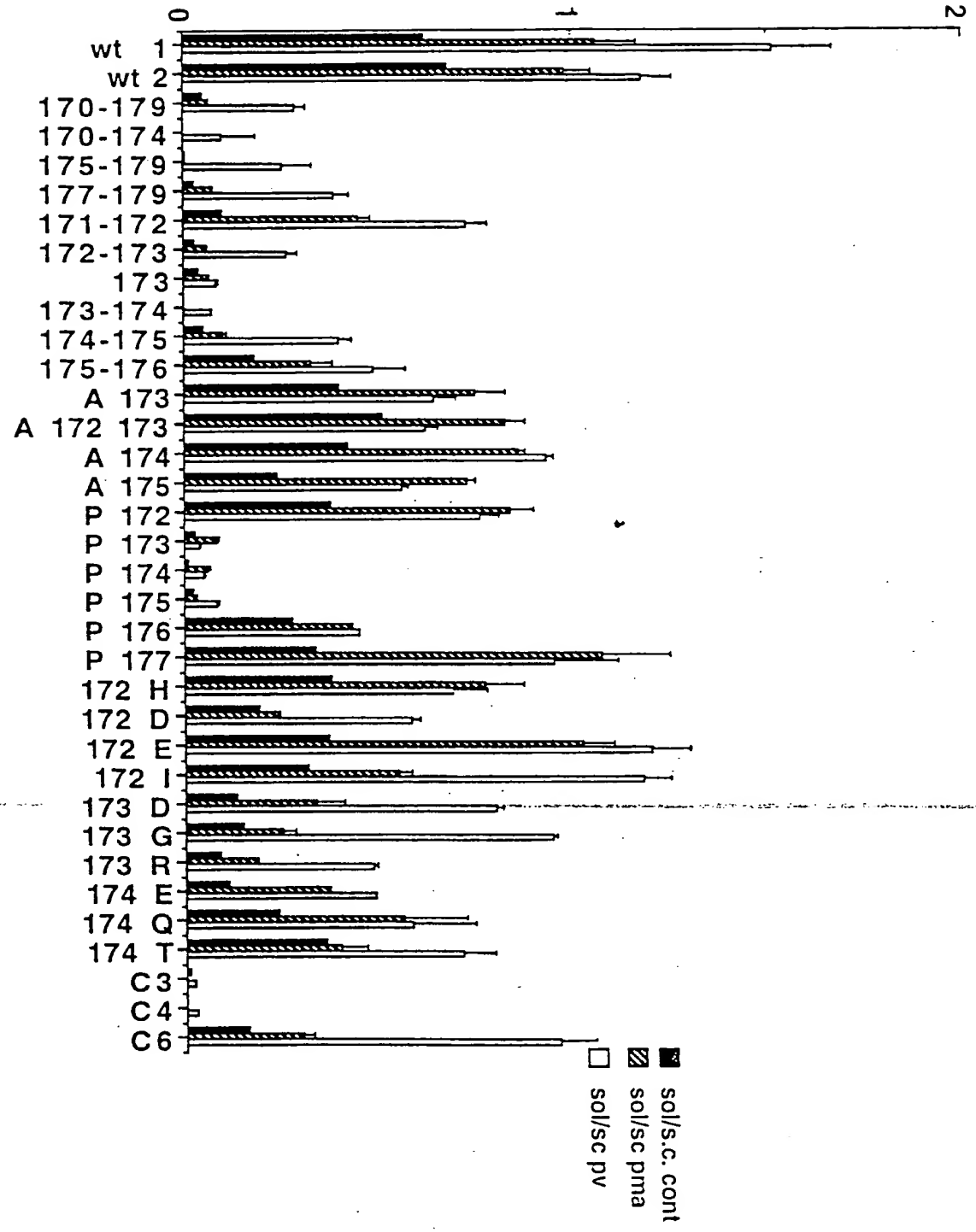

Figure 9

BNSDOCID: <EP___0657536A1_I_>

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 11 6018

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	JOURNAL OF BIOLOGICAL CHEMISTRY., vol.5, no.28, 5 October 1991, BALTIMORE, MD US pages 18846 - 18853 F. PORTEU ET AL 'Human neutrophil elastase releases a ligand-binding fragment from the 75-kDa tumor necrosis factor (TNF) receptor'	1,2, 4-19,23, 24	C12N15/57 C12N9/64 C12N1/21 C12N5/10 A61K38/48 C07K14/415 C07K16/40 A61K39/395
Y	* the whole document *	3	
X	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol.34, March 1993 page 441 L. ANGELO AND L. OWEN-SCHAUB 'Elastatinal, A-PMSF, and pepstatin inhibit p80 tumor necrosis factor (TNF-R) shedding in activated human lymphocytes'	1,4-19	
Y	*abstract 2632*	3	
X	BIOCHEMISTRY, vol.30, no.42, 22 October 1991 pages 10065 - 10074 M. EHLERS AND J.RIORDAN 'Membrane proteins with soluble counterparts: Role of proteolysis in the release of transmembrane proteins' * page 10069, right column, last paragraph - page 10071 *	1,2, 4-19,23, 24	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N C07K A61K
X	EUROPEAN JOURNAL OF CELL BIOLOGY, vol.58, 1992 pages 307 - 312 U. GULLBERG ET AL 'Involvement of an Asn/Val cleavage site in the production of a soluble form of human tumor necrosis factor (TNF) receptor.'	1,2, 4-19,23, 24	
Y	* the whole document *	3	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	6 February 1995	Van der Schaal, C

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 11 6018

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	CHEMICAL ABSTRACTS, vol. 112, no. 7, 12 February 1990, Columbus, Ohio, US; abstract no. 51017, J. HEIMBACH ET AL 'Affinity purification of the HIV-1 protease' page 309 ; * abstract * & BIOCHEM. BIOPHYS. RES. COMMUN., vol.164, no.3, 1989 pages 955 - 960	3	
A	PROC. R. SOC. LOND. B, vol.245, 1991 pages 115 - 119 C. HWANG ET AL 'A 20 amino acid synthetic peptide of a region from the 55 kDa human TNF receptor inhibits cytolytic and binding activities of recombinant human tumor necrosis factor in vitro' * the whole document *	20-22	
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.87, no.19, October 1990, WASHINGTON US pages 7380 - 7384 P. GRAY ET AL 'Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein'		TECHNICAL FIELDS SEARCHED (Int.Cl.6)
P,X	EP-A-0 568 925 (YEDA RESEARCH AND DEVELOMENT) 10 November 1993	1,2, 4-19, 22-24	
Y	* the whole document *	3	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	6 February 1995	Van der Schaal, C

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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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